

The synthesis and purification of 2'-carboxy-D-arabinitol 1-phosphate, a natural inhibitor of ribulose 1,5-bisphosphate carboxylase, investigated by ^{31}P n.m.r.

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2'-Carboxy-D-arabinitol 1-phosphate (2CA1P), a natural inhibitor of ribulose 1,5-bisphosphate carboxylase was synthesized from 2'-carboxy-D-arabinitol 1,5-bisphosphate (2CABP). The selective dephosphorylation of 2CABP with either acid phosphatase or alkaline phosphatase was investigated by using ^{31}P n.m.r. The n.m.r. spectra of the progress of the reactions indicated that both phosphatases preferentially removed the 5-phosphate from the bisphosphate. After the consumption of all of the bisphosphate, alkaline phosphatase generated a mixture of 2'-carboxy-D-arabinitol 1- and 5-monophosphates in the ratio of about 4:1, along with P_i . The enzyme also hydrolysed the monophosphates to 2'-carboxyarabinitol, thus decreasing the yield of 2CA1P further. In contrast, acid phosphatase catalysed almost quantitative conversion of 2CABP into 2CA1P, preferring to hydrolyse only the 5-phosphate. In either case, separation of the 2CA1P from P_i or other products of enzymic hydrolysis was readily accomplished by conventional ion-exchange chromatography or h.p.l.c.

INTRODUCTION

During periods of decreased light, ribulose 1,5-bisphosphate carboxylase (rubisco, EC 4.1.1.39) in some plants suffers a significant decline in activity [1–5] due to the accumulation of the inhibitor 2'-carboxy-D-arabinitol 1-phosphate [(2-C-phosphohydroxymethyl)-D-ribonate; 2CA1P] in the chloroplast [6,7]. The inhibitor binds so avidly to the activated form of rubisco, with a dissociation constant of 3.2×10^{-8} M, that isolation of the enzyme from dark-exposed leaves into solutions containing saturating concentrations of the activating cofactors, CO_2 and Mg^{2+} , does not restore the activity to that obtained for enzyme extracted from light-treated leaf material. Nevertheless, exposure of the inhibited enzyme to high concentrations of competing ions such as SO_4^{2-} , followed by gel filtration [5], or treatment with phosphatases [7], restored the activity.

These methods were the first to demonstrate unequivocally that the activity of rubisco was diurnally regulated through binding a phosphate-containing compound. Investigations of the effects of ribulose 1,5-bisphosphate (ribulose- P_2) analogues and competitive inhibitors of turnover on the activation state of the carboxylase indicated that there may be a role for effectors that favour the activated form of the enzyme *in vivo* [8–10]. Many stabilized the activated enzyme- CO_2 - Mg^{2+} ternary complex at physiological concentrations of CO_2 and Mg^{2+} . Unfortunately, none of the effectors investigated apparently attain an appropriate concentration in the chloroplast to assign them this function unequivocally. Therefore, the observation that the specific activity of rubisco was dependent on the

presence of a phosphate-containing inhibitor, was the first indication that a natural analogue might indeed possess this function. Furthermore, that the changes in concentration of the inhibitor depend on the amount of irradiance of the plant [3–5], provides an alternative means of light regulation of rubisco and thus photosynthetic C-fixation.

Detailed ^{13}C and ^1H n.m.r. along with mass spectroscopy of the natural inhibitor isolated and purified from two plant sources, potato (*Solanum tuberosum*) and French Bean (*Phaseolus vulgaris*), established the structure as 2CA1P, an analogue of the hydrated *gem-diol* form of 2'-carboxy-3-oxoarabinitol-1,5-bisphosphate, the transition-state intermediate of the carboxylase reaction [11]. The successful identification of 2CA1P as the inhibitor relied initially on natural sources, but the structure was only unequivocally assigned by synthesis of the monophosphate. The synthesis was based on the dephosphorylation of the tight-binding inhibitor 2'-carboxy-D-arabinitol 1,5-bisphosphate (2CABP). One method recognized that alkaline phosphatase showed some discrimination between the phosphate groups of 2CABP and combined this with ion-exchange chromatography to obtain an enriched preparation of 2CA1P [6]. A second method used rubisco to selectively bind the monophosphate in preference to the other products of the hydrolysis and then exploit the enzyme as a means of purifying the inhibitor [7].

In the present study the methods required to obtain acceptable quantities of synthesized 2CA1P from 2CABP have been substantially improved as a prelude to investigating the metabolic processes that produce and degrade the inhibitor.

Abbreviations used: 2CA1P, 2'-carboxy-D-arabinitol 1-phosphate; 2CABP, 2'-carboxy-D-arabinitol 1,5-bisphosphate; rubisco, ribulose 1,5-bisphosphate carboxylase; ribulose- P_2 , ribulose 1,5-bisphosphate; 2CRBP, 2'-carboxyarabinitol 1,5-bisphosphate.

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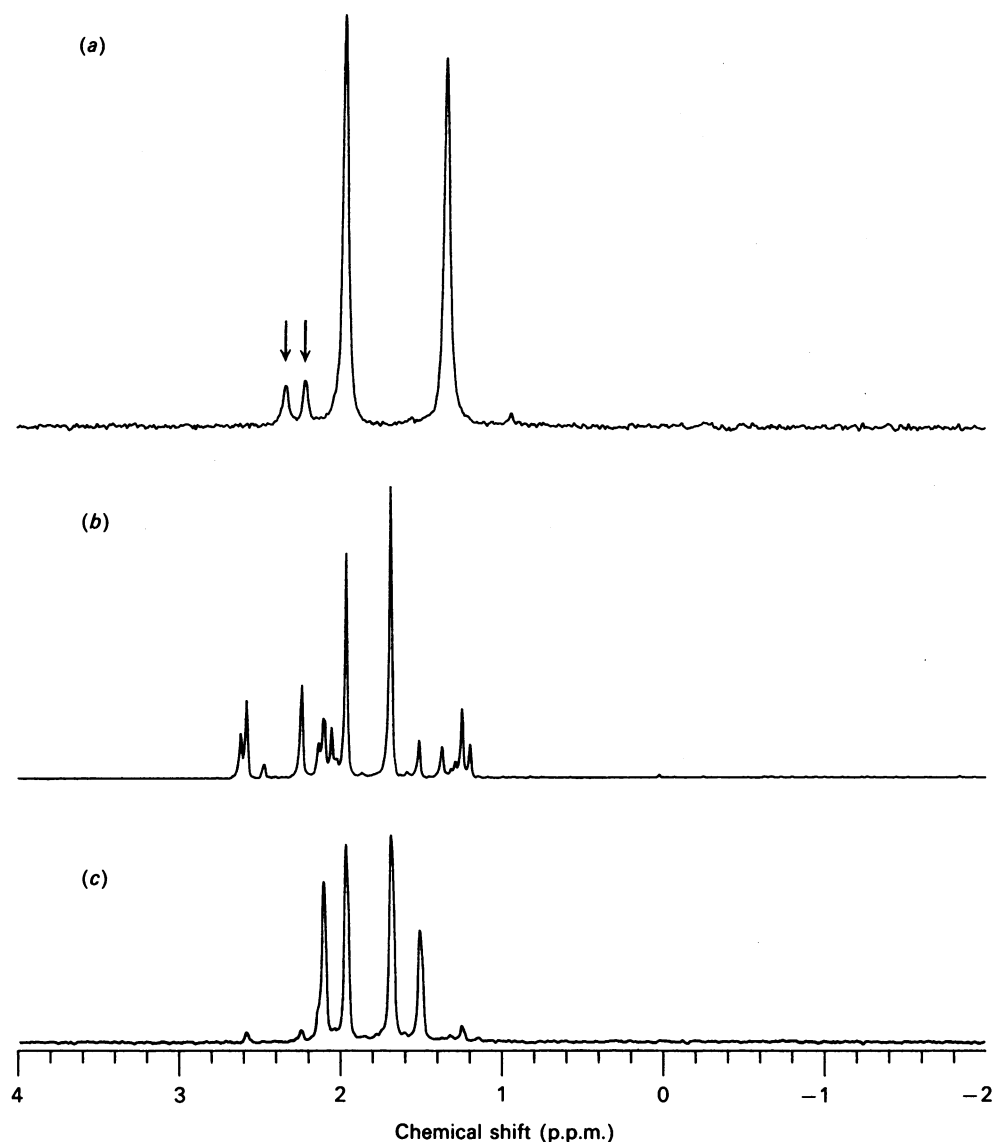


Fig. 1. Synthesis of the two 2'-carboxypentitol bisphosphates from ribulose- P_2

(a) Shows the ^{31}P -n.m.r. spectrum of ribulose- P_2 in Tris/HCl buffer, pH 8.3. The positions of the resonances due to the hydrated species are indicated to lower field. The spectrum in (b) shows the effect of allowing the bisphosphate to react for 24 h with an equimolar concentration of cyanide. The two resonances at 1.96 p.p.m. and 1.68 p.p.m. due to 2CABP dominate the spectrum. The spectrum in (c) shows the final mixture of 2'-carboxyribitol 1,5-bisphosphate (2CRBP) and 2CABP was obtained after 36 h of reaction with cyanide.

MATERIALS AND METHODS

Phosphatases were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. Acid phosphatase was a preparation from potato and alkaline phosphatase was from bovine liver. Rubisco was purified from spinach (*Spinacia oleracea*) leaves as previously described [12].

Preparation of phosphate esters

Ribulose- P_2 was synthesized from ribose 5-phosphate as described by Weissbach *et al.* [13]. Ribulose- P_2 was purified by ion-exchange chromatography on a Dowex 1 (200–400 mesh) resin column equilibrated with 3 mM-HCl and developed with a linear NaCl gradient (0–0.4 M).

The 2'-carboxypentitol 1,5-bisphosphates were synthesized from purified ribulose- P_2 by reaction with cyanide, using the procedures described by Pierce *et al.* [15], except that the solutions were maintained at pH 8.3 with 0.1 M-Tris/HCl or triethanolamine buffers. The resulting mixture of 2'-carboxypentitol stereoisomers was freed of excess cyanide and buffer cations by addition of Dowex 50 (H^+ form) and lactonized by rotary evaporation at acid pH. The two isomers were resolved by ion-exchange chromatography using a Dowex 1 (AG8; 200–400 mesh) resin column (3.2 cm \times 24 cm) essentially as described for ribulose- P_2 . The separated bisphosphate lactones were recovered by precipitation as the barium salts by addition of a 30-fold molar excess of barium acetate and then an equal volume of ethanol. The white precipitate was collected by centrifugation on a

bench-top centrifuge [3000 rev./min (r_{av} , 14 cm) for 15 min], resuspended in water containing Dowex 50 resin (H^+ form) to remove Ba^{2+} and solubilize the bisphosphates. The resulting slurry was passed over a short column of the Dowex 50 resin and the bisphosphates collected with two bed volumes of water. The final concentration of the material was adjusted by rotary evaporation. The lactones were converted to the free-acid forms by saponification at elevated pH (see the Results section).

Synthesis of the various monophosphates was achieved enzymically by treating the bisphosphates with acid or alkaline phosphatases. Acid phosphatase hydrolysis of 2CABP was performed at pH 5.7 (in 20 mM-sodium formate). In the case of alkaline phosphatase, the pH was adjusted to 8.3 (20 mM-Tris/HCl) before the addition of enzyme.

N.m.r. spectra

^{31}P -n.m.r. spectra were collected with a Nicolet 360 MHz machine set at 141.2 MHz. Samples were shimmed using a deuterium lock and broad-band decoupling was applied. The sample volume in 12 mm tubes was routinely 5 ml containing 1 ml of 2H_2O . Sample quantities were generally about 50 μ mol with respect to phosphate, and those spectra generated by the addition of enzyme required the accumulation of about 100 pulses. Assignment of the phosphate resonances in the n.m.r. spectrum of the 2'-carboxypentitol bisphosphates was by selective enrichment of either the 1- or 5-phosphate group with ^{17}O [19].

H.p.l.c. analysis

H.p.l.c. analysis of the composition of the monophosphates was achieved using a Whatman Partisil Sax-10 ion-exchange analytical column on a HP1090 instrument (Hewlett-Packard). The column was developed with a pH 3.8 ammonium phosphate buffer gradient (10–30 mM). The monophosphates were detected at 215 nm by using a diode-array spectrophotometer. The

monophosphates were readily identified by the inhibition of rubisco activity [6]. Details of other column-chromatographic techniques are given in the Results section.

RESULTS

The usual conditions for generating the 2'-carboxypentitol 1,5-bisphosphates from ribulose- P_2 involve the addition of, at neutral pH, a slight molar excess of cyanide, allowing the solution to readjust to about 9.5. Unfortunately, even at moderately alkaline pH values, ribulose- P_2 rearranges by eliminating the 1-phosphate to form a dicarbonyl compound, with the ability to interconvert further [14] or potentially react with cyanide. Under the conditions chosen here, the pH was buffered at 8.3 to minimize this unwanted reaction in favour of generating only the 2'-nitrile addition compounds.

Cyanide reacts with ribulose- P_2 at either the *re* or *si* face of the 2-carbonyl, generating two stereoisomeric pentitols, initially in the form of nitriles that ultimately hydrolyse at acid pH values to the 2'-carboxy derivatives [16]. The ^{31}P -n.m.r. spectrum in Fig. 1 shows that all of the ribulose- P_2 is converted into product after 36 h at room temperature and pH 8.3, although it is clear from Fig. 1(b) that the arabinitol isomer with resonances at 1.68 p.p.m. and 1.96 p.p.m. forms more quickly than ribitol.

At acid pH values, both isomers cyclize to the lactones, in which form they can be readily resolved. Unfortunately, lactonization at pH 2.0 is relatively slow unless water is removed. This was achieved initially by rotary evaporation followed by storage in a vacuum desiccator overnight with P_2O_5 . Fig. 2 shows the separation of the 2'-[^{14}C]carboxypentitol bisphosphate lactones synthesized from 1 mmol of ribulose- P_2 (by reaction with [^{14}C]cyanide) by ion-exchange chromatography. The distribution of the radioactivity suggested that the synthesis produced about equal amounts of the pentitol lactones. The peaks at 0.17 M- and 0.21 M-salt are due to a small

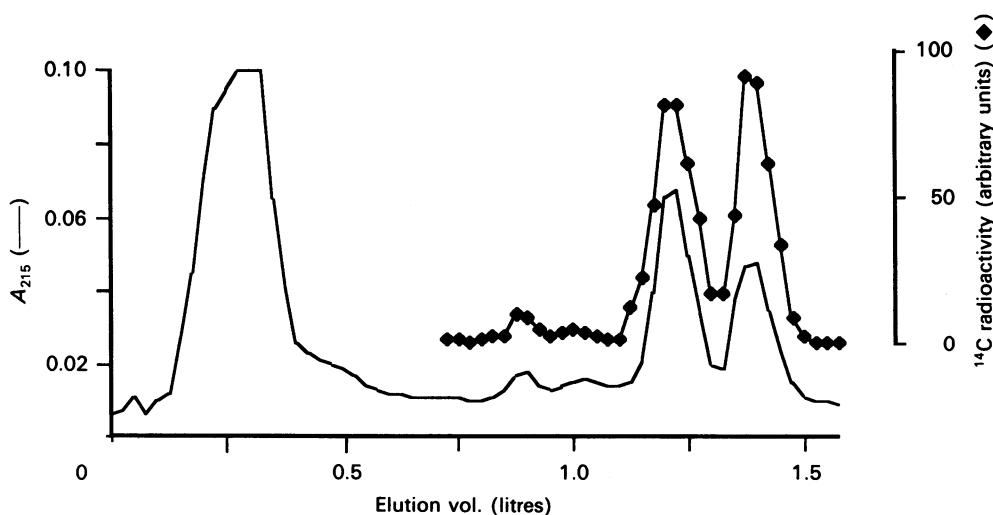


Fig. 2. Separation of the two stereoisomeric bisphosphates

The Dowex 1 column was equilibrated with 3 mM-HCl and developed with a linear (0–0.4 M) NaCl gradient. The peaks at 0.17 M (0.75 litre) and 0.21 M (1.0 litre) are due to 2CABP and 2CRBP free acids remaining after lactonization. 2CRBP and 2CABP lactones are eluted at 0.25 M (1.2 litres)- and 0.28 M (1.4 litres)-salt respectively.

amount of the bisphosphate that has not lactonized during the extensive dehydration.

As the open-chain free-acid form, 2CABP is bound by rubisco essentially irreversibly ($K_d = 10^{-11}$), because it resembles the 2'-carboxy six-carbon intermediate of the carboxylase reaction [11]. As the lactone, inhibition is decreased by some five orders of magnitude and is similar to that obtained for other substrate analogues. Conversion of the lactone into the open form generates protons that, without adequate buffering, decrease the pH and thus slow, or even stop, the process at neutral pH values. Saponification proceeds with a $t_{1/2}$ of about 2 h with solutions buffered at pH 8.3 in $\frac{1}{2}$ Tris/HCl or triethanolamine. At a pH of 10.9, buffered by carbonate, the ring-opening is complete in about 10 min (results not shown).

Enzymic generation of the monophosphates from the bisphosphates

The course of the enzymic hydrolysis of the phosphate groups of 2CABP was observed directly by using ^{31}P n.m.r. Potentially two monophosphates, 2CA1P and 2CA5P, might be generated from the bisphosphate by a non-specific phosphatase, and the structures of these are shown in Fig. 3, along with the parent compound, 2CABP. Fig. 4 shows the fate of the phosphates of 2CABP (open-chain form) with time after the addition of acid phosphatase at pH 5.7, as monitored by n.m.r. spectroscopy. Both the resonances due to 2CABP (1-phosphate at 1.23 and the 5-phosphate at 1.65 p.p.m.) decline at the same rate, with the simultaneous appearance (also at the same rate) of two other resonances. The first at 1.35 p.p.m. is due to the formation of 2CA1P. The other is P_i (set at 0 p.p.m.) released from the 5-position of the bisphosphate. The discrimination between the 1- and 5-phosphates by acid phosphatase ensures essentially quantitative conversion of the bisphosphate into only 2CA1P, i.e. the P_i and 2CA1P integrals are almost identical. Furthermore, the subsequent hydrolysis of 2CA1P by the phosphatase is extremely slow, with none detected over the course of the experiment.

For comparison, Fig. 4(f) is the spectrum obtained after the treatment of 2CABP with alkaline phosphatase at pH 8.3. During hydrolysis with this enzyme, the 2CABP resonances are replaced by three species. At this pH, the 2CA1P resonance shifts downfield to 1.78 p.p.m. relative to P_i . At 2.02 p.p.m. is an additional monophosphate due to the generation of 2CA5P. The amount of 2CA5P corresponds to about 25% of the total monophosphates. The production of 2CA5P by

alkaline phosphatase indicates that the 1-phosphate is susceptible to hydrolysis and thus the ultimate fate of both monophosphates is complete dephosphorylation to 2'-carboxyarabinitol. The end result is, therefore, that the P_i peak becomes the only detectable n.m.r. species.

The difference in the ratio of the products of the two phosphatase activities was clearly demonstrated using analytical h.p.l.c. The phosphatase activity was stopped by decreasing the pH below 2.0 by treating the solutions with Dowex 50 resin (H^+ form). The 2'-carboxylic acid products were then lactonized by rotary evaporation. The retention times of the two monophosphates was established by their effect on the carboxylase activity of rubisco. As the open-chain form 2CA1P is a superior inhibitor by at least two orders of magnitude than 2CA5P. The h.p.l.c. elution profiles shown in Fig. 5(a) and (b) are from the n.m.r. samples after phosphatase treatment (Figs. 4e and 4f respectively).

The 2CA1P generated by acid phosphatase is contaminated with P_i and potentially any unhydrolysed 2CABP, which must be removed if the monophosphate is to be used as an inhibitor of the carboxylase. Chromatography on a Dowex 1 column (formate form) developed with a formic acid gradient readily separates the P_i from the inhibitor as shown in Fig. 6. In these conditions 2CABP remains bound to the column and is only eluted by washing with 0.5 M- Cl^- ion. The monophosphate was retrieved free of Na^+ and formate ions by treatment with Dowex 50 (H^+ form) and rotary evaporation. The resulting product is again in the form of a lactone that must be saponified at elevated pH values to be used as an effective rubisco inhibitor.

DISCUSSION

The evident discrimination between the 1- and 5-phosphates of 2CABP by the phosphatases used in this investigation has been exploited as a means of generating (particularly with acid phosphatase) the natural inhibitor of rubisco, 2CA1P. This has been achieved without requiring enzyme intervention to extract 2CA1P from a mixture of the monophosphates [7] or the need to remove other contaminating phosphate esters [6]. The nature of the discrimination between the 1- and 5-phosphates was not studied in detail, but was found to occur for the carboxyribitol as well as the carboxyarabinitol bisphosphate stereoisomers.

Clearly, the differences may lie in the presence of a charged carboxy group at the 2'-position in both molecules. Indeed the same discrimination was evident

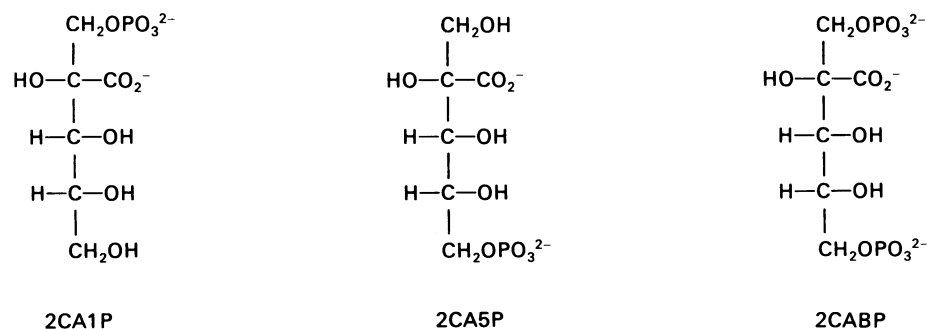


Fig. 3. Structures of the free-acid forms of the monophosphates generated from 2CABP

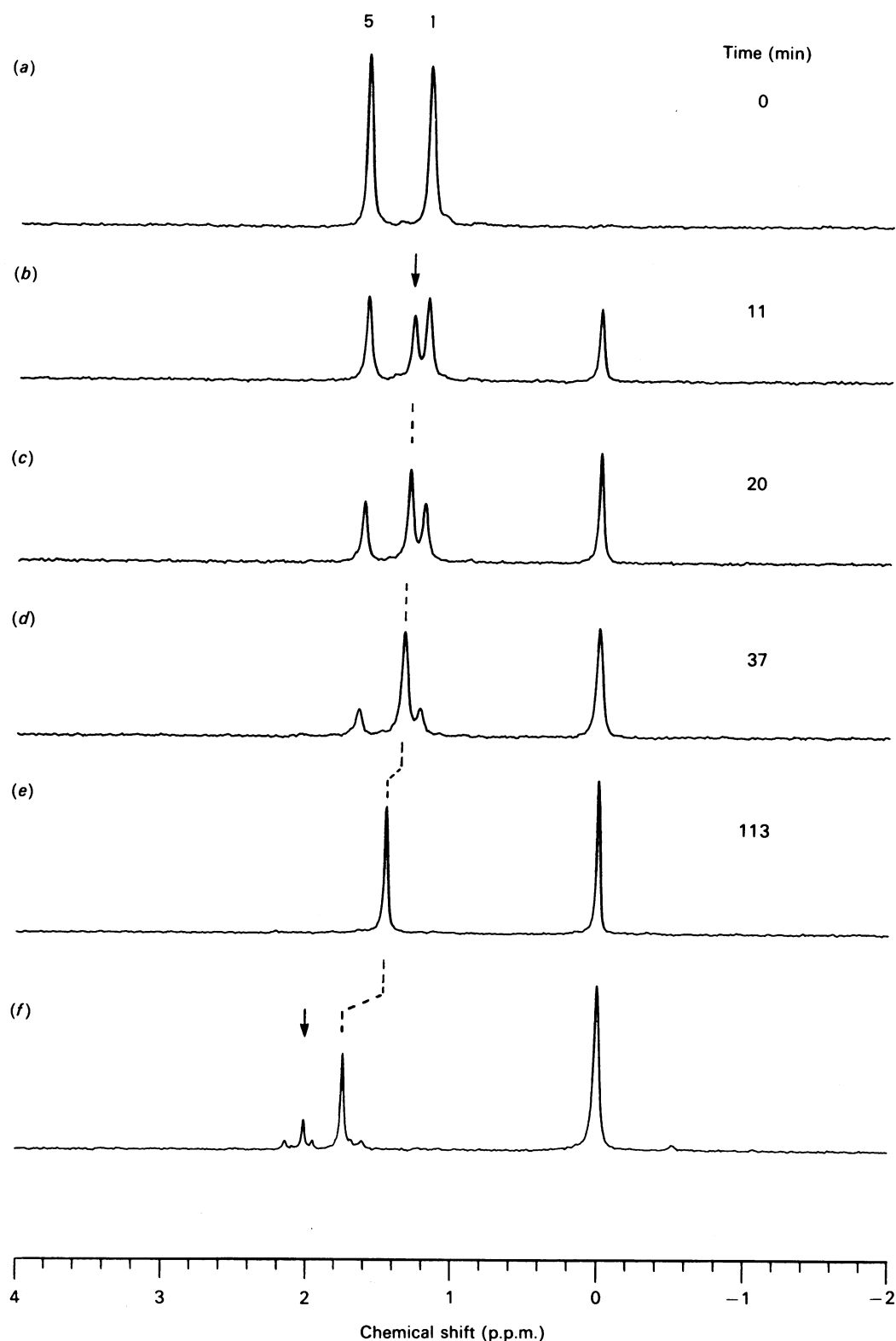


Fig. 4. Synthesis of 2CA1P from 2CABP by treatment with phosphatases

The spectra shown in (a)–(e) are the time courses of the hydrolysis of 2CABP to 2CA1P by acid phosphatase at pH 5.7. (a) shows the spectrum of the starting compound, 2CABP (free-acid form) at pH 5.7 with the resonances of the 1- and 5-phosphate groups indicated. Spectra (b)–(d) were collected at 11, 20 and 37 min after the addition of acid phosphatase (5 units). The appearance of the 1-phosphate group of 2CA1P (arrowed in b) is shown as the reaction proceeds. After 110 min, more enzyme (8 units) was added to drive the reaction to completion, and the spectrum in (e) was recorded 3 min later. The downfield shift in position of the 2CA1P resonance in (e) is due to a 0.1 pH unit increase in pH on the addition of the neutral enzyme solution. For comparison, (f) shows the mixture of products obtained after treatment of 2CABP with alkaline phosphatase (10 units, 120 min) at pH 8.3. The resonance at 2.02 p.p.m. is due to the formation of 2CA5P (arrowed) and those at 1.63 p.p.m. and 1.98 p.p.m. from unconverted 2CABP. At pH 8.3 all the resonances are shifted downfield relative to the position of P_i by about 0.4 p.p.m.

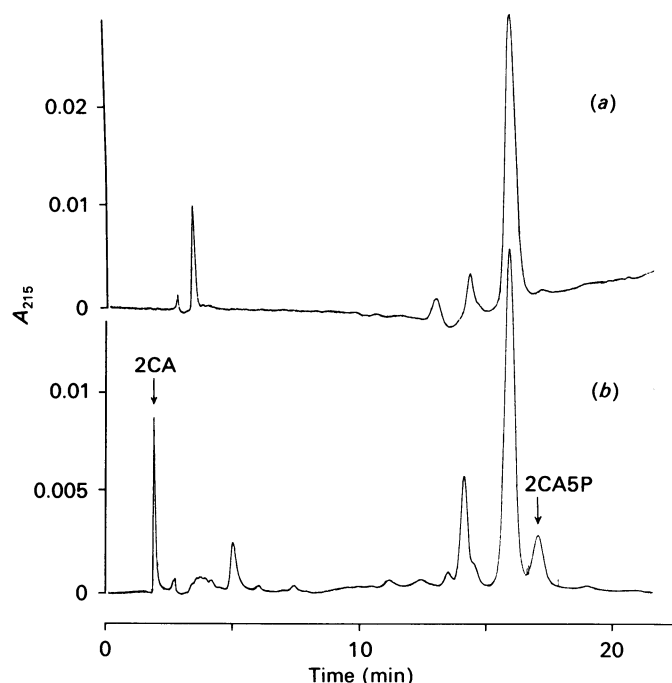


Fig. 5. Analysis and purification of the monophosphates synthesized from 2CABP

The h.p.l.c. chromatograph shown in (a) is an analysis of the products generated from acid phosphatase treatment of 2CABP compared with that in (b) obtained after hydrolysis of the bisphosphate with alkaline phosphatase. The peak eluted after 16 min is 2CA1P lactone. 2CA5P and 2-carboxyarabinitol (2CA) lactones are indicated; the other peaks originate from the buffers used for the hydrolysis and the ammonium phosphate buffer eluent. The gradient was linear from 10 mM to 30 mM-ammonium phosphate, pH 3.8, over 25 min.

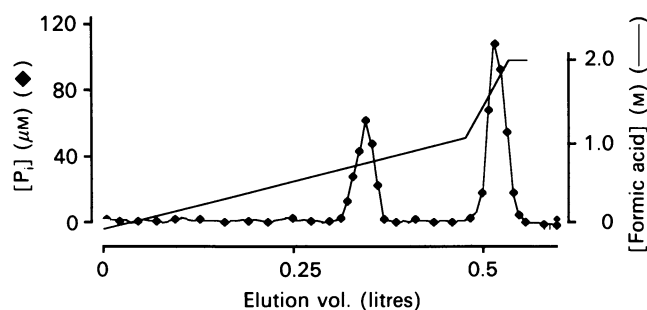


Fig. 6. Isolation of 2CA1P free of P_i

The Dowex 1 (formate form) column was equilibrated with water and developed with a linear formic acid gradient (0–1.0 M). P_i was eluted at 0.7 M-formate. The monophosphate was recovered after stepping the gradient to 2 M-formic acid. The P_i was detected by using a Malachite Green colorimetric assay [17]. If the monophosphate was not radioactively labelled, the phosphate was released by incubation with alkaline phosphatase at 37 °C and also detected colorimetrically.

with 4'-carboxyarabinitol bisphosphate as substrate (results not shown). Further support for these assumptions was obtained from an investigation of the hydrolysis of the phosphate groups of 2CABP lactone, in which the 2'-carboxy group is effectively blocked by

esterification with the 4-hydroxy group of the sugar backbone. Loss of a significant amount of the discrimination was evident from the amount of P_i generated during the reaction (results not shown). Furthermore, 2CA1P (now as the lactone) was no longer resistant to hydrolysis by acid phosphatase and at pH 5.7 was degraded.

There is clearly some difference between the specificity of acid phosphatase compared with alkaline phosphatase for the two phosphates, the former apparently being unable to remove the 1-phosphate group of the open-chain form of the bisphosphates, whereas alkaline phosphatase removes both, albeit at different rates. The difference in preference might be due to the lower pH at which the acid phosphatase reaction is carried out, e.g. the 1-phosphate is about 80% protonated at pH 5.7, which combined with a charge and/or steric effect of the 2'-carboxy group may ensure its resistance to removal.

Irrespective of the mechanistic differences between the two phosphatases, the selectivity of the acidophilic enzyme has provided a means of producing 2CA1P in good yield. The ability to synthesize 2CA1P with appropriate isotopic enrichment combined with some of the analytical techniques described above (see also [18]) should prove useful in attempting to determine the metabolic processes responsible for the diurnal changes observed for this inhibitor *in vivo*.

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